JOURNAL OF VIROLOGY, Aug. 2005, p. 10660–10671 Vol. 79, No. 16
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.16.10660–10671.2005


# The Ebola Virus Genomic Replication Promoter Is Bipartite and Follows the Rule of Six

Michael Weik,[1,2]¶ Sven Enterlein,[1]¶ Kathrin Schlenz,[1,3] and Elke Mühlberger[1]*

*Department of Virology, Philipps University Marburg, Robert-Koch-Str.17, 35037 Marburg, Germany[1]; Dade Behring, Marburg GmbH, Marburg, Germany[2]; and Beiersdorf AG, Hamburg, Germany[3]*

Received 22 March 2005/Accepted 29 April 2005

**In this work we investigated the *cis*-acting signals involved in replication of Ebola virus (EBOV) genomic RNA. A set of mingenomes with mutant 3′ ends were generated and used in a reconstituted replication and transcription system. Our results suggest that the EBOV genomic replication promoter is bipartite, consisting of a first element located within the leader region of the genome and a second, downstream element separated by a spacer region. While proper spacing of the two promoter elements is a prerequisite for replication, the nucleotide sequence of the spacer is not important. Replication activity was only observed when six or a multiple of six nucleotides were deleted or inserted, while all other changes in length abolished replication completely. These data indicate that the EBOV replication promoter obeys the rule of six, although the genome length is not divisible by six. The second promoter element is located in the 3′ nontranslated region of the first gene and consists of eight $UN_5$ hexamer repeats, where N is any nucleotide. However, three consecutive hexamers, which could be located anywhere within the promoter element, were sufficient to support replication as long as the hexameric phase was preserved. By using chemical modification assays, we could demonstrate that nucleotides 5 to 44 of the EBOV leader are involved in the formation of a stable secondary structure. Formation of the RNA stem-loop occurred independently of the presence of the trailer, indicating that a panhandle structure is not formed between the 3′ and 5′ ends.**

Ebola virus (EBOV) and Marburg virus are members of the family *Filoviridae*, which belongs to the order *Mononegavirales*. Ebola viruses are divided into four genera, Zaire, Sudan, Ivory Coast, and Reston (26). All filoviruses cause severe hemorrhagic fevers in humans and nonhuman primates, with fatality rates of up to 90% (36), except for EBOV Reston, which seems to be apathogenic for humans (18, 23). EBOV genomes consist of a nonsegmented, single-stranded RNA in negative orientation (NNS) of about 19 kb in length. Seven genes encoding eight proteins are arranged in a linear order. Short nontranscribed regions are located at the extreme 3′ and 5′ ends, called the leader and the trailer, respectively (Fig. 1A). Compared to other members of the *Mononegavirales*, filovirus (and henipavirus) genes have unusually long nontranslated regions (NTRs) flanking the open reading frames. Thus, the start codon of the first EBOV gene, the NP gene, is located at nucleotide positions 470 to 472, and the stop codon of the last gene, the L gene, is found 742 nucleotides upstream of the extreme 5′ end. Naturally occurring defective interfering EBOV particles revealed that 155 nucleotides at the 3′ terminus and 176 nucleotides of the 5′ terminus are sufficient for replication (5).

Four viral proteins, NP, VP35, VP30, and L, are associated with the viral RNA forming the nucleocapsid (1). While the nucleoprotein NP tightly encapsidates the viral RNA, the catalytic subunit of the viral polymerase, L, and the polymerase cofactor VP35 constitute the viral polymerase complex. The fourth nucleocapsid protein, VP30, is involved in the formation of the nucleocapsid complex and necessary for rescue of full-length recombinant EBOV (34, 46). Interestingly, VP30 is an activator of EBOV transcription in a reconstituted minigenome system but not necessary for replication (3, 27, 32, 50). Also, this fourth nucleocapsid protein is unique to filoviruses and pneumoviruses within the *Mononegavirales* (2, 14).

Due to the similar genome organization among all members of the *Mononegavirales*, transcription and replication are assumed to follow common mechanisms. During EBOV transcription, the viral polymerase transcribes the seven genes to produce eight monocistronic mRNA species which are capped and polyadenylated (31, 45, 50). Interestingly, transcription of the glycoprotein gene is accompanied by mRNA editing, a phenomenon also found with the phosphoprotein gene of the members of the *Paramyxovirinae* subfamily (39, 43, 45). During replication, the polymerase enters the genome at the 3′ end (including the leader), which contains a *cis*-acting promoter region, and generates a complementary copy of the genome, the antigenome (19). This antigenome is also readily encapsidated and used as a template to yield progeny negative-stranded RNA. Then, the complement of the genomic 5′ end (cTrailer) serves as a promoter for replication with significantly enhanced efficiency compared to the leader, as shown for the closely related Marburg virus (29). Leader and cTrailer show a high degree of homology within the first 50 nucleotides but differ in the ability to support transcription.

Within the order *Mononegavirales*, two structures of replication promoters have been described. First, the promoter is located entirely within the leader region, as shown for vesicular stomatitis virus, a member of the *Rhabdoviridae*, and respiratory syncytial virus (RSV), belonging to the *Pneumovirinae*

* Corresponding author. Mailing address: Department of Virology, Philipps University Marburg, Robert-Koch-Str.17, 35037 Marburg, Germany. Phone: 6421-2864525. Fax: 6421-2865482. E-mail: muehlber@staff.uni-marburg.de.

¶ Both authors contributed equally to this work.

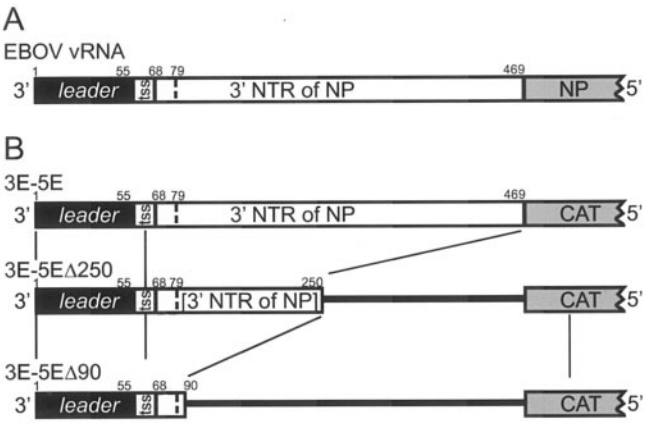


FIG. 1. Schematic drawing of the 3′ region of EBOV RNA and the derived minigenomes. (A) Known elements within the EBOV genomic 3′ terminus. The 3′ genomic end of EBOV RNA contains a nontranscribed region from nucleotides 1 to 55 (leader; black) followed by the transcription start signal of the NP gene (tss; nucleotides 56 to 67). The transcription start signal is involved in formation of a hairpin loop (light gray; nucleotides 56 to 78). Nucleotides 56 to 469 are not translated; translation of NP (dark gray) starts with the AUG at position 470. (B) Overview of the 3′ regions of minigenomes. 3E-5E contains the leader, the entire 3′ nontranslated region (NTR) of the NP gene, and the CAT gene instead of viral genes. Deletions were introduced upstream of the reporter gene to truncate 5′ sequences of the 3′ NTR. Plasmid 3E-5EΔ250 served as a template for the other mutants created in this work. Construct 3E-5EΔ90 is shown as a representative of the deletion mutants. Numbers refer to the genome sequence of EBOV Zaire, strain Mayinga (GenBank accession number AF086833).

subfamily of the *Paramyxoviridae* (21, 25). Second, the promoter consists of two distinct elements which are separated by a spacer region with unimportant sequence. However, this spacer has to be of defined length, as shown for Sendai virus and other members of the *Paramyxovirinae* (24, 33, 42, 49). The known bipartite promoters consist of a stretch of about 30 nucleotides located at the 3′ end of the genome and a second well-defined element within the nontranslated region of the first (genomic promoter) or the last gene (antigenomic promoter), respectively. A common feature of all NNS viruses containing bipartite replication promoters is that the total number of nucleotides in the genome is divisible by six (rule of six) (6, 13, 20). However, this is not the case for rhabdoviruses, pneumoviruses, and filoviruses (5, 35, 38).

In this paper we analyzed the secondary structure at the 3′ end of the EBOV Zaire genome and its role in the replication of minigenomic RNA. Further experiments were performed to elucidate the structure of the replication promoter. We found that the promoter is bipartite and important motifs follow the rule of six.

## MATERIALS AND METHODS

**Cell lines and viruses.** HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The human hepatoma cell line Huh-T7, constitutively expressing the T7 RNA polymerase (kindly provided by V. Gaussmüller, Department of Medical Molecular Biology, University of Lübeck, Lübeck, Germany), was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1 mg/ml geneticin. BSR T7/5 cells (derived from a BHK-21 cell line), also constitutively expressing the T7 RNA polymerase (kindly provided by K. K. Conzelmann, Max von Pettenkofer Institute and Gene Center, Munich, Germany), were cultured as described by Buchholz et al. (4). Chicken embryo fibroblasts were used to grow the recombinant vaccinia virus MVA-T7 containing the T7 RNA polymerase gene (41). For this work, the EBOV Zaire strain Mayinga was used. Nucleotide numbers refer to GenBank accession number AF086833.

**Cloning of the minigenomes.** The EBOV-specific minigenome 3E-5E (32) or its derivative 3E-5EΔ250 (50) was used as the template to generate all negative-sense mutant constructs; positive-sense mutants were based on 3E-5E(+) (50). These minigenomes contain the EBOV leader, the entire (3E-5E) or a part (3E-5EΔ250) of the 3′ NTR of the NP gene, the chloramphenicol acetyltransferase (CAT) gene as a reporter gene, followed by the 5′ NTR of the EBOV L gene and the trailer (Fig. 1B). Substitutions of up to 4 nucleotides were introduced using the QuikChange Mutagenesis kit (Stratagene).

For construction of deletion mutants of the 3′ end, PCR fragments flanked by an RsrII and an NdeI restriction site were generated from 3E-5E. 3E-5E was digested with RsrII and NdeI to remove EBOV-specific sequences upstream of the CAT gene. Ligation of the PCR fragments with the vector resulted in 3′-truncated minigenomes. For creation of random substitution mutants, an SpeI (nucleotides $GAGGAA_{56-61}$→ACUAGU) and a SacII restriction site (nucleotides $GAAAUU_{84-89}$→CCGGCC) were introduced into 3E-5EΔ250 by several rounds of QuikChange mutagenesis to yield 3E-5EΔ250 SpeI/SacII. Introduction of the SpeI site resulted in destruction of the transcription start signal of the NP gene.

Two oligonucleotides were annealed to create fragments containing the randomized sequence (Table 1). The generation of 3E-5EΔ250x84-130 involved two steps: 3E-5EΔ250 SpeI/SacII was first cut with SpeI and SacII, and the annealed oligonucleotides 1080 and 1081 were inserted. The resulting construct was then digested with XhoI and MluI and annealed oligonucleotides 1082 and 1083 were inserted. The SpeI site was then removed in all constructs that were used for determination of the spacer length to restore the transcription start signal. Constructs 3E-5EΔ250+6, 3E-5EΔ250+9, and 3E-5EΔ250+12 were generated by inserting annealed oligonucleotides (Table 1) between nucleotide 73 and 74 of 3E-5EΔ250 SacII digested with SexAI. Plasmid 3E-5EΔ250×55–90 was digested with SpeI and subsequently treated with Klenow fragment to obtain 3E-5EΔ250×55–90+4, which contained an additional 4 nucleotides (GATC). In vitro mutagenesis PCR was used to generate the deletion mutants using 3E-5EΔ250 SacII as the template. Table 2 gives an overview of the resulting sequences.

**Infection and transfection of eukaryotic cells.** Replication of EBOV minigenomes was assayed by using a plasmid-based reconstituted replication/transcription system (32). In this system, expression of the nucleocapsid proteins and generation of the minigenomic RNA are driven by the T7 RNA polymerase. For expression of the T7 RNA polymerase, either HeLa cells were infected with the MVA-T7 virus or constitutively expressing cell lines were used (BSR T7/5 or Huh-T7 cells). When we compared the results obtained in the different systems, we did not observe remarkable differences.

**Infection and transfection of HeLa cells.** HeLa cells were seeded in six-well plates to a density of 60% and infected with MVA-T7 virus at a multiplicity of infection of 5 PFU per cell. At 1 h postinfection, the following amounts of plasmids were used for transfection using Lipofectamine (Invitrogen): 1.0 μg $pT/L_{EBO}$, 0.5 μg $pT/NP_{EBO}$, 0.5 μg $pT/VP35_{EBO}$, 0.1 μg $pT/VP30_{EBO}$, and 2 μg of the respective minigenomic DNA. At 2 days postinfection, cells were lysed and subjected to RNA analysis.

**Transfection of BSR T7/5 and Huh-T7 cells.** BSR T7/5 and Huh-T7 cells were grown in six-well plates to 60 to 70% confluence and transfected using FUGENE 6 (Roche Molecular Applied Science). For transfection, 1.0 μg minigenome, 1.0 μg $pT/L_{EBO}$, 0.5 μg $pT/NP_{EBO}$, 0.5 μg $pT/VP35_{EBO}$, 0.1 μg $pT/VP30_{EBO}$, and 0.5 μg of pC-T7/Pol (expressing the T7 RNA polymerase [34]; kindly provided by T. Takimoto, St. Jude Children's Research Hospital, Memphis, Tenn., and Y. Kawaoka, University of Wisconsin, Madison) were used. Transfection was carried out as described by Modrof et al. (28). At 2 days after transfection, cells were lysed in the appropriate buffer and analyzed for CAT expression or RNA synthesis.

**Isolation and detection of replicated RNA.** Transfected cells were washed twice with phosphate-buffered saline and lysed under mild conditions in 200 μl of micrococcal nuclease buffer (10 mM NaCl, 10 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 5% Triton X-100, 0.3% sodium deoxycholate, 10 mM $CaCl_2$). The lysate was sheared 10 times through a 24-gauge needle and sonicated for 60 s. Cell debris was removed by brief centrifugation (5 min at 500 × *g*) and the supernatant was incubated with 51 U of micrococcal nuclease (MBI Fermentas) for 70 min at 33°C. Afterwards, RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The recovered RNA was then analyzed by Northern blotting. The positive-stranded replicative interme-

TABLE 1. Sequences of the primers used for cloning of substitution, insertion, and deletion mutants. Indicated restriction sites were used for insertion and/or subsequent cloning steps.

| Primer | Sequence (5′→3′) | Restriction site(s) | Construct |
|---|---|---|---|
| 475 | GGTGGACCTTTTTCGGATCCGCAAA | SpeI, SacII | 3E-5EΔ250 × 55–90 |
| 476 | CTAGTTTGCGGATCCGAAAAAGGTCCACCGC | | |
| 868 | CCAGGTGTGATTACAGTAACAATCGACCAGATCTAATAACGTGTCCGC | SexAI, SacII | 3E-5EΔ250 × 84-110 |
| 869 | GCGGACACGTTATTAGATCTGGTCGATTGTTACTGTAATCACACCTGG | | |
| 1070 | CCAGGTGTGATTAATTGCCATCGCGACCAGATCTAATAACGTGTCCGC | SpeI, SacII | 3E-5EΔ250 × 84-120 |
| 1071 | GGACACGTTATTAGATCTGGTCGCGATGGCAATTAATCACA | | |
| 1080 | CCAGGTGTGATTAATTGCCATCGCGACCAGATCTAATAACGTGTCCGC | SpeI, MluI, SacII | 3E-5EΔ250 × 84-120′ |
| 1081 | GGACACGTTATTAGATCTGGTCGCGATGGCAATTAATCACA | | |
| 1082 | TCGAGCTTTGTGATGTGGCTCTGAAACAAACCACCGCA | XhoI, MluI | 3E-5EΔ250 × 84-130 |
| 1083 | CGCGTGCGGTGGTTTGTTTCAGAGCCACATCACAAAGC | | |
| 1121 | GGAATGAGAGGATCATTTAAATTATTAATC | SexAI | 3E-5EΔ250 +6 |
| 1122 | GATTAATAATTTAAATGATCCTCTCATTCC | | |
| 1182 | GGAATGAGAGGATCACGCTTTAAATTATTAATC | SexAI | 3E-5EΔ250 +9 |
| 1183 | GATTAATAATTTAAAGCGTGATCCTCTCATTCC | | |
| 1184 | GAGAGGATCACGCATCTTTAAATTATTAATC | SexAI | 3E-5EΔ250 +12 |
| 1185 | GATTAATAATTTAAAGATGCGTGATCCTCTC | | |

diate was detected using a negative-stranded, digoxigenin-labeled riboprobe directed against the CAT gene (29).

**CAT assay and CAT ELISA.** BSR T7/5 or Huh-T7 cells were transfected as described above. Cells were washed twice with phosphate-buffered saline and lysed in 150 μl of reporter lysis buffer (Promega). Two days posttransfection, CAT assays were performed using a standard protocol. Quantification of processed chloramphenicol was done with a Bioimager Analyzer (Fuji BAS-1000) and the Raytest TINA software. CAT enzyme-linked immunosorbent assays (ELISAs) were carried out using the CAT ELISA kit (Roche Applied Science) following the manual's instructions. Samples were quantified using the supplied standards.

**In vitro transcription and chemical modification assay.** Plasmid 3E-5E(+) was linearized either with SalI prior to in vitro transcription to generate a positive-sense runoff transcript containing the complete minigenome or with NdeI to generate a runoff transcript containing only the first 472 nucleotides of the EBOV Zaire genome. Transcription was performed with the AmpliScribe T7 kit (Epicenter) according to the manufacturer's instructions. RNA secondary-structure formation was investigated by chemical modification assays with dimethyl sulfoxide (51) to modify A and C residues and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimid metho-*p*-toluensulfonate (CMCT) to modify G and U residues as described elsewhere (50). Modified RNA species were analyzed by primer extension. Briefly, 1 μg or 50 μg RNA, respectively, was incubated with either dimethyl sulfoxide or CMCT, precipitated, and subjected to reverse transcription (SuperScript II reverse transcriptase, Invitrogen) using a $^{32}$P-labeled primer binding to nucleotides 60 to 90 of the EBOV leader. In parallel, the DNA template 3E-5E(+) was radioactively sequenced with the same primer using the T7 sequencing kit (Amersham Biosciences). Reaction products were separated on an 8% denaturing polyacrylamide gel and the dried gel was exposed to a Bioimager plate (Fuji). The plates were visualized with the Bioimager analyzer (Fuji BAS-1000) and Raytest TINA software.

## RESULTS

**Ebola virus leader does not interact with the trailer.** Sequence comparison of the 3′ and 5′ end of the EBOV genome revealed stretches of complementary nucleotides within each region as well as between the two regions. Leader and trailer could therefore form stable secondary structures themselves

TABLE 2. Sequence of the EBOV 3′ end of the spacer mutants starting at position 55[a]

| Construct | Sequence around modification (3′→5′) |
|---|---|
| 3E-5EΔ250 −12 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$ACUU U AAAUA$_{80}$U AGCCU U AAA$_{90}$UUU AACUU U A$_{100}$ACAA U GACAU$_{110}$ |
| 3E-5EΔ250 −9 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$GUAA C UUUAA$_{80}$A UAUAG C CUU$_{90}$AAA UUUAA C U$_{100}$UUAA C AAUGA$_{110}$ |
| 3E-5EΔ250 −6 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAG U AACUU$_{80}$U AAAUA U AGC$_{90}$CU U AAAUU U A$_{100}$ACUU U AACAA$_{110}$ |
| 3E-5EΔ250 −5 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAA G UAACU$_{80}$U UAAAU A UAG$_{90}$CC U UAAAU U U$_{100}$AACU U UAACA$_{110}$ |
| 3E-5EΔ250 −3 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAA G AGUAA$_{80}$C UUUAA A UAU$_{90}$AGC CUUAA A U$_{100}$UUAA C UUUAA$_{110}$ |
| 3E-5EΔ250 −2 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAG A GAGUA$_{80}$A CUUUA A AUA$_{90}$UAG CCUUA A A$_{100}$UUUA A CUUUA$_{110}$ |
| 3E-5EΔ250 −1 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAG G AGAGU$_{80}$A ACUUU A AAU$_{90}$AUA GCCUU A A$_{100}$AUUU A ACUUU$_{110}$ |
| 3E-5EΔ250 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAAA G GAGAG$_{80}$U AACUU U AAA$_{90}$UAU AGCCU U A$_{100}$AAUU U AACUU$_{110}$ |
| 3E-5EΔ250 +1 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**U** AGGAGA$_{80}$G UAACU U UAA$_{90}$AUA UAGCC U U$_{100}$AAAU U UAACU$_{110}$ |
| 3E-5EΔ250 +2 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**CU** AGGAG$_{80}$A GUAAC U UUA$_{90}$AAU AUAGC C U$_{100}$UAAA U UUAAC$_{110}$ |
| 3E-5EΔ250 +3 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**ACU** AGGA$_{80}$G AGUAA C UUU$_{90}$AAA UAUAG C C$_{100}$UUAA A UUUAA$_{110}$ |
| 3E-5EΔ250 +5 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**UUACU** AG$_{80}$G AGAGU A ACU$_{90}$UUA AAUAU AG$_{100}$CCUU A AAUUU$_{110}$ |
| 3E-5EΔ250 +6 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**UUUACU** A$_{80}$G GAGAG U AAC$_{90}$UUU AAAUA U A$_{100}$GCCU U AAAUU$_{110}$ |
| 3E-5EΔ250 +9 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**UUUCGCA**$_{80}$**C**U AGGAG AGU$_{90}$AAC UUUAA A U$_{100}$AUAG C CUUAA$_{110}$ |
| 3E-5EΔ250 +12 | C U CCU$_{60}$UC U AAUUA U UU$_{70}$AAA**UUUCUAC**$_{80}$**G**CACU AGGAG$_{90}$AGU AACUU U A$_{100}$AAUA U AGCCU$_{110}$ |

[a] Boxes indicate positions at which repetitive U residues are found. The inserted sequence is represented in bold.

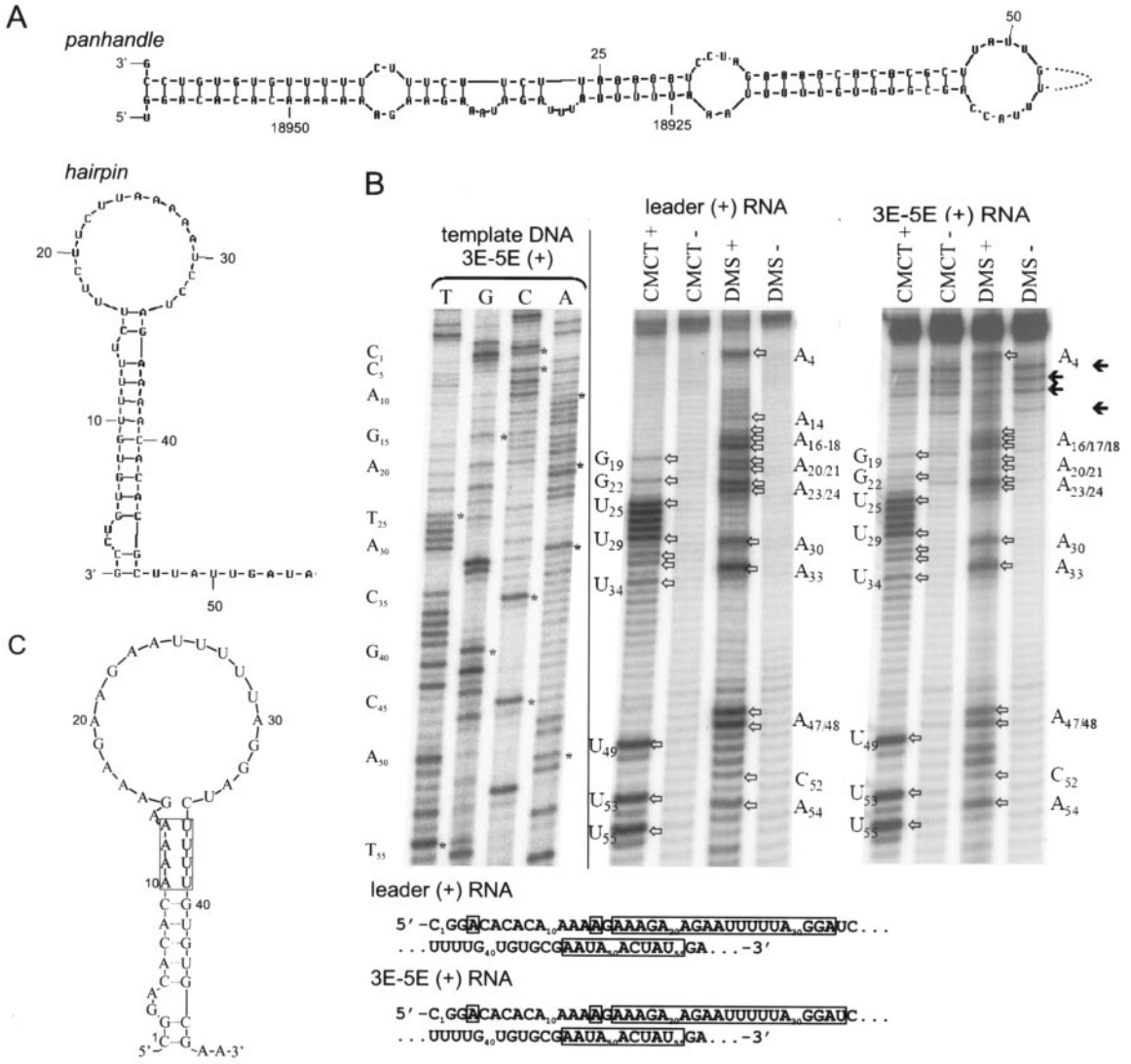


FIG. 2. Analysis of the secondary structure formed within the first 50 nucleotides of the EBOV genome. (A) Computer-predicted secondary structures of the genomic ends. (B) Autoradiographs of in vitro-transcribed and chemically modified 3E-5E(+) and leader(+) RNAs in direct comparison to elucidate the nature of the secondary structure. Treatment of RNA with dimethyl sulfoxide leads to modification of unpaired A and C residues, whereas treatment with CMCT modifies unpaired U and G residues. These modified bases lead to termination of reverse transcription with a radioactively labeled primer, and the resulting bands were resolved on a sequencing gel along with the sequence of the 3E-5E(+) template (left lanes). Lanes DMS− and CMCT− indicate reverse transcription products of untreated RNA template as a background control. Asterisks in the template lanes refer to bands belonging to the nucleotides placed on the left-hand side. Open arrows indicate modified nucleotides, while solid arrows point to unspecific bands most likely due to generation of cDNA on short RNA fragments. The sequences at the bottom refer to the positive-sense RNA. Modified nucleotides are boxed and show no difference between the trailer being present, in 3E-5E(+), or not, in leader(+). Note that the cDNA fragments synthesized from modified RNA are displaced by 1 nucleotide relative to the sequencing lanes. (C) Sketch of the secondary structure as deduced from the chemical modification assay. Again, the RNA sequence is numbered according to the positive strand of the EBOV leader. The area in which mutations were introduced in further experiments is highlighted with a gray box.

(hairpin loop) or with each other (panhandle). In silico analysis predicted a stable secondary structure within the first 50 nucleotides of the EBOV Zaire genome involving direct pairing of nucleotides 4 to 15 with 33 to 43 (Fig. 2A). Alternatively, the highly conserved 3′- and 5′-terminal nucleotides could interact, leading to the formation of a panhandle structure (Fig. 2A).

To investigate whether the hairpin loop is present or leader and trailer instead form a panhandle, the actual structure was determined by chemical modification assays of in vitro-transcribed RNAs. Two different RNAs were synthesized: one represented a positive-stranded analogue of the minigenome 3E-5E and were therefore termed 3E-5E(+), comprising the first 472 nucleotides of the EBOV genome, a CAT reporter gene, and the last 729 nucleotides of the EBOV genome. The other RNA was designated EBOV leader(+). It contained only the first 472 nucleotides of the 3′ end in the positive orientation without the CAT gene and trailer. After in vitro transcription, the RNA was subjected to chemical modification with CMCT and dimethyl sulfoxide. Dimethyl sulfoxide specif-

ically modifies unpaired A and C residues and CMCT specifically modifies unpaired U and G residues. The modified RNAs were reverse transcribed, whereby progress of the reverse transcriptase was inhibited by modified nucleotides leading to termination. Termination products were then separated on a denaturing urea-polyacrylamide gel along with the sequencing reaction of the template.

Comparison of the modified bases clearly indicated no difference between the two samples (Fig. 2B). According to the results of the chemical modification assays, nucleotides 5 to 15 interacted with nucleotides 35 to 44, forming a stem region (Fig. 2C). Formation of a stable hairpin loop within the first 50 nucleotides of the EBOV Zaire genome is therefore independent of the presence of the trailer. This also leads to the presumption that panhandle structures between the genomic ends are not formed. However, it has to be considered that all modifications were made on a nonencapsidated RNA of positive orientation. Efforts to perform chemical modification assays with EBOV nucleocapsids were not successful, presumably due to too small an amount of isolated RNA.

**First 55 nucleotides of the EBOV 3′ terminus contain important signals for replication.** Since the results of the chemical modification assays provided evidence that nucleotides 5 to 44 of the EBOV leader are involved in RNA secondary-structure formation, the question arose whether the secondary structure of the hairpin loop and/or the primary sequence is important for replication. To address this question, an EBOV-specific minigenome system, in which replication and transcription are reconstituted by plasmid-supplied minigenomic RNA and nucleocapsid proteins, was used. Two minigenomic mutants were designed and checked for their ability to be replicated by the nucleocapsid proteins. Huh-T7 cells were transfected with plasmids encoding the EBOV nucleocapsid proteins NP, VP35, VP30, and L. Additionally, a reference minigenome (3E-5EΔ250) or a mutated minigenome was transfected.

Construct 3E-5EΔ250×10–13 contained four exchanges of A to U residues (nucleotides 10 to 13), which are involved in stem-loop formation (Fig. 2B and C). To restore the disrupted base pairings, compensatory exchanges of nucleotides 36 to 39 were introduced to yield construct 3E-5EΔ250restore_2°. After 2 days, cells were harvested and tested for either CAT activity or replicated RNA. It has to be noted that replication (in the absence of transcription) does not lead to CAT gene expression. However, CAT activity does not only reflect transcription but has been shown to be dependent on replication of the minigenome as well (32). As shown in Fig. 3A, none of the mutant constructs was able to support transcription of the CAT gene. Since this might be due to the lack of sufficient template, the mutants were assayed for replication. Replicated RNA is encapsidated and hence nuclease resistant. Northern blot analysis of RNA treated with micrococcal nuclease verified that no replication had occurred (Fig. 3B). These findings led to the conclusion that secondary-structure formation per se is not sufficient to support replication. However, it cannot be ruled out that both the primary sequence and the secondary structure are important for replication activity.

The transcription start signal of the NP gene, comprising nucleotides 56 to 67, is located 12 nucleotides downstream of the above-mentioned hairpin loop. Next, we wanted to address the question of whether the nucleotides located at the 5′ end of the leader between the putative secondary structure and the transcription start signal of the NP gene were essential for replication. Three sets of substitutions should clarify the role of nucleotides 44 to 55. In each mutant, three adjacent nucleotides were substituted: nucleotides 44 to 46, 50 to 52, and 53 to 55. The resulting plasmids (3E-5E×44–46, 3E-5E×50–52, and 3E-5E×53–55, respectively) were analyzed for their ability to support replication by using the reconstituted replication and transcription system.

To this end, HeLa cells were infected with MVA-T7 and subsequently transfected with plasmids coding for the nucleocapsid proteins and the mutated minigenomes. At 30 h posttransfection, cells were lysed and replicated RNA was isolated. Northern hybridization against the positive-sense replicative intermediate did not reveal any replicated RNA (Fig. 3C). Taken together, it could be shown that the first 55 nucleotides, i.e., the entire leader region up to the transcription start signal of the first gene, are important for replication. Although our results indicate that the leader might interact with itself to form a stable hairpin loop, this structure alone seems not to be sufficient to support replication.

**Replication signals extend into the NP gene.** The 3′ end of the EBOV minigenome 3E-5E is 469 nucleotides in length and comprises the leader and the 3′ NTR of the NP gene. To determine the minimum sequence requirement for replication of minigenomic RNA, the 3′ NTR of the NP gene of minigenome 3E-5E was consecutively truncated from the 5′ end (Fig. 1B). Deletions were introduced by using PCR and standard molecular biology techniques. A set of 16 deletion mutants were generated, ranging from 52 to 351 nucleotides remaining at the 3′ end (Fig. 1B).

To get an idea about the ability of these mutants to support replication, a quantitative CAT ELISA was performed. Although this is a method to analyze transcription of the reporter gene, it can be used indirectly to estimate replication (32). Briefly, BSR T7/5 cells were transfected with the plasmids encoding the nucleocapsid proteins and the mutated minigenomes or 3E-5E. At 2 days after transfection, cells were lysed and normalized amounts of all lysates were used in a standard CAT ELISA.

For deletions up to nucleotide 150, levels of translated CAT protein were similar to that of 3E-5E (Fig. 4A, left-hand side). CAT expression decreased dramatically with mutant 3E-5EΔ108 to drop below detectable levels when 94 or fewer nucleotides remained (Fig. 4A, left-hand side). Further mutants containing 140 to 121 remaining nucleotides were created and tested in a CAT assay for replication and transcription activity. The CAT activity of this set of mutants was similar to that of the wild type (Fig. 4A, right-hand side). After this first impression of replication- and transcription-competent constructs, 14 truncated minigenomes were tested directly for replicated RNA using Northern blot analysis. Although, due to the method employed, a direct quantification of the replicated RNA was not possible, the tendency of decreasing replication activity with larger deletions is clearly visible (Fig. 4B). While deletions up to nucleotide 96 always yielded detectable levels of RNA, further deletions showed no or only traces of replicated RNA in repeated experiments. We therefore concluded

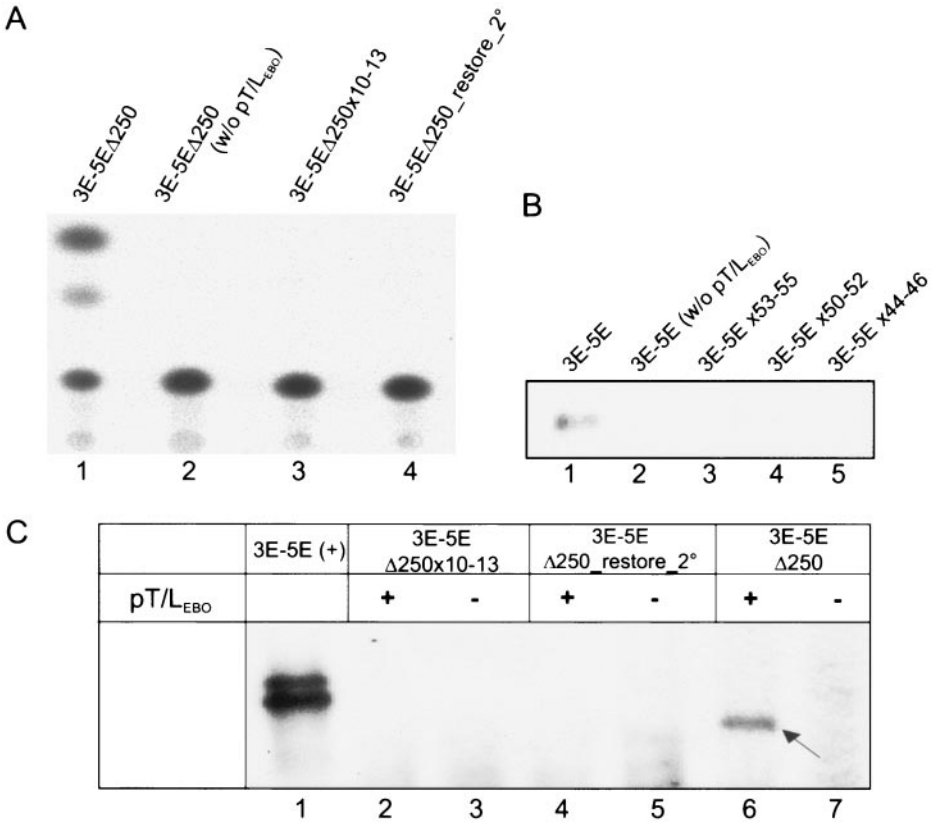


FIG. 3. Nucleotide substitutions within the leader region of the EBOV genome interfere with replication activity. (A) Effects of disruption and restoration of the stem-loop structure within nucleotides 1 to 50 of the EBOV leader: coupled replication/transcription assay. Huh-T7 cells were transfected with plasmids pT/VP30$_{EBO}$, pT/VP35$_{EBO}$, pT/NP$_{EBO}$, and pT/L$_{EBO}$ encoding the nucleocapsid proteins and the (mutated) minigenome. At 2 days posttransfection, cells were lysed and the lysates were subjected to a CAT assay. (B) Effects of disruption and restoration of the stem-loop structure within nucleotides 1 to 50 of the EBOV leader: Northern blot analysis of replicated RNA. Huh-T7 cells were transfected with plasmids encoding the nucleocapsid proteins and the minigenomes as indicated above the lanes. At 2 days posttransfection, cells were lysed under mild conditions and subjected to micrococcal nuclease treatment. Replicated RNA was isolated and detected by Northern blot technique using a digoxigenin-labeled riboprobe directed against the positive-strand replication intermediate (32). The first lane, 3E-5E(+), is in vitro-transcribed RNA used as a hybridization control and size marker; the two bands are due to inefficient cleavage of the ribozyme. Minigenome 3E-5EΔ250 ×10–13 contains four A→U exchanges, disrupting the stem structure depicted in Fig. 2B. Compensatory substitutions of nucleotides 36 to 39 were introduced in plasmid 3E-5EΔ250restore_2°. The arrow indicates replicated RNA of the positive control. (C) Effect of substitutions of nucleotides 44 to 55. Minigenomes which contained each of three substitutions (see Materials and Methods for details) were assayed for replication. HeLa cells were infected with MVA-T7 and transfected as described above. At 30 h posttransfection, encapsidated RNA was isolated and subjected to Northern blot analysis as described above. As a negative control, plasmid pT/L$_{EBO}$ encoding the L protein was omitted (lane w/o pT/L$_{EBO}$).

that important signals for replication must reside within the transcribed region of the NP gene upstream of nucleotide 96.

**EBOV replication promoter is bipartite.** After locating important stretches of sequence within the first 55 nucleotides and upstream of nucleotide 96, it was of interest to investigate whether the EBOV replication promoter is a single element, as described for RSV (9), or segmented in two parts, as was shown for Sendai virus (42). To address this question, the previously described construct 3E-5EΔ250 was modified so that two additional restriction sites, SpeI at nucleotides 56 to 61 within the transcription start signal of the NP gene and SacII at nucleotides 84 to 89, were introduced. After verification that the substitutions did not affect replication competence (data not shown), these two sites were used to replace nucleotides 55 to 90 by either a random sequence of the same length or a random sequence containing four additional nucleotides. The sequences were carefully checked for the absence of motifs similar to promoter elements found in the genomes of other members of the *Mononegavirales* (see Discussion). The resulting minigenomes, 3E-5EΔ250 ×55–90 and 3E-5EΔ250 ×55–90+4, along with the plasmids encoding the nucleocapsid proteins, were used to transfect Huh-T7 cells. At 2 days posttransfection, replicated RNA was isolated and detected by the Northern blot technique.

Surprisingly, exchange of 36 nucleotides spanning the region between nucleotides 55 to 90 did not affect replication dramatically (Fig. 5A, lane 3). However, when the random sequence was elongated by four additional nucleotides, replication activity could not be detected (Fig. 5A, lane 7). These results suggested that (i) the EBOV replication promoter might be bipartite and (ii) proper spacing of the two elements might be important for efficient replication.

To narrow down the putative second promoter element, further substitutions were introduced to change nucleotides 84

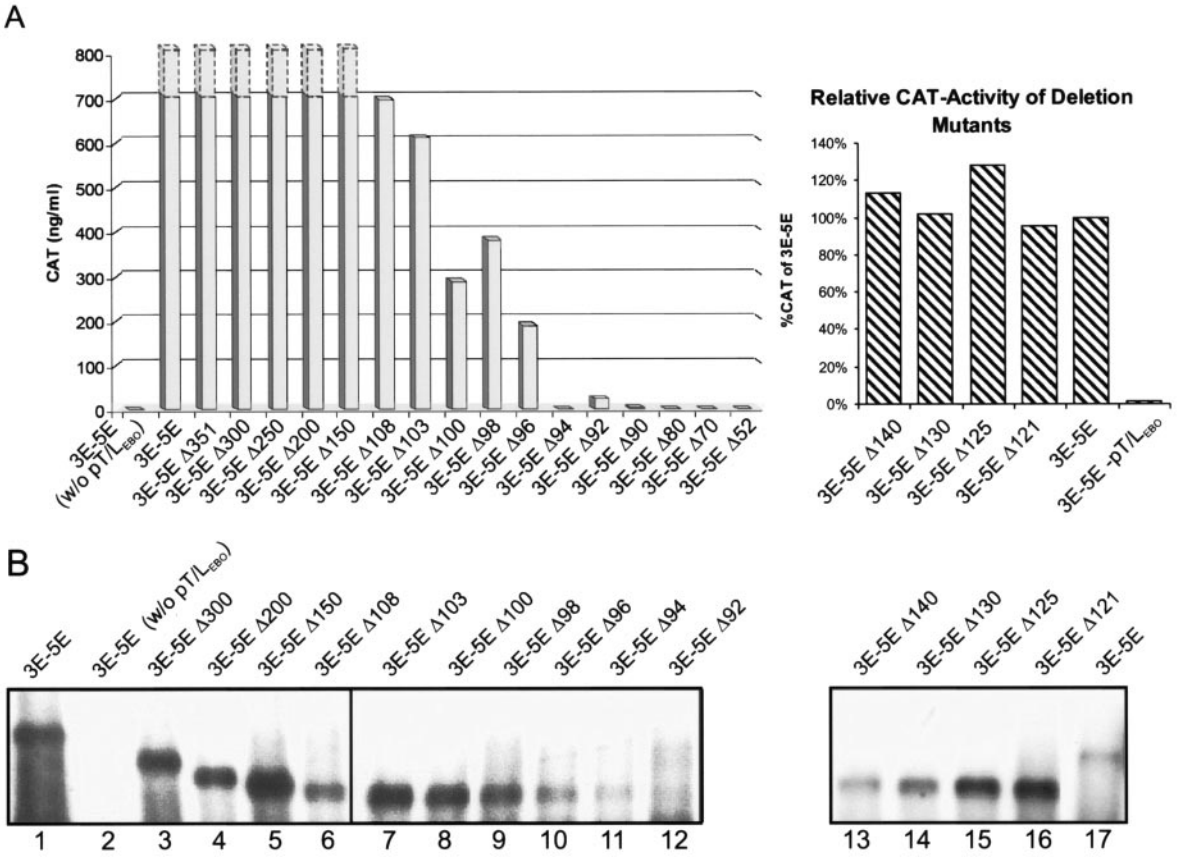


FIG. 4. Determination of the minimal sequence requirement for efficient replication. Minigenome 3E-5E was truncated upstream of the CAT gene, i.e., in the 3′ region of the EBOV genome (Fig. 1B). The resulting mutants were tested for replication in the artificial replication/transcription assay. (A) CAT ELISA and CAT assay. BSR T7/5 cells were transfected with plasmids encoding the nucleocapsid proteins $L_{EBO}$, $VP35_{EBO}$, $VP30_{EBO}$, and $NP_{EBO}$ and the minigenomes as indicated. At 24 h posttransfection, cells were lysed, and normalized amounts of the lysates were either assayed for CAT expression using a CAT-ELISA or subjected to the CAT assay. (B) Northern blot analysis. HeLa cells were infected with MVA-T7 and transfected with the same set of plasmids as described above. At 2 days posttransfection, cells were lysed, and the lysates were treated with micrococcal nuclease. Replicated RNA was purified and analyzed by Northern blot assay. The number in the constructs' names reflects the remaining nucleotides of the EBOV 3′ end. In both assays, 3E-5E (w/o $pT/L_{EBO}$), where $pT/L_{EBO}$ was omitted from transfection, served as a negative control.

to 110, 84 to 120, and 84 to 130. For these mutants, however, the previously introduced SpeI restriction site was resubstituted to allow transcriptional analysis. Huh-T7 cells were transfected with the plasmids coding for the nucleocapsid proteins and the respective minigenomes and harvested 2 days posttransfection. Using CAT activity or the amount of CAT protein (ELISA) as an estimate of replication, replication/transcription activity could only be detected for mutant 3E-5EΔ250 ×84–110 but not for minigenomes 3E-5EΔ250 ×84–120 and 5EΔ250 ×84–130 (data not shown). Again, these results were verified by direct detection of replicated RNA products in a Northern blot assay. Huh-T7 cells were transfected with the plasmids coding for the nucleocapsid proteins and the respective minigenomes and harvested 2 days posttransfection. Encapsidated RNA was isolated and subjected to Northern blot analysis. When nucleotides 84 to 110 were substituted, replication activity could be detected but was reduced (Fig. 5A, lane 4). When an additional 10 or 20 nucleotides were changed, replication of the mutant minigenomes was not observed (Fig. 5A, lanes 5 and 6).

At first glance, these results seemed to be in conflict with the findings obtained from the truncation mutants described above. A possible explanation for this discrepancy arose during further experiments, when the structure of the EBOV promoter was analyzed in more detail (see Fig. 7 and Discussion). Nevertheless, these data already suggested a bipartite structure of the EBOV replication promoter similar to that found in Sendai virus, since a stretch of at least 56 nucleotides could be exchanged internally without abolishing replication as long as the length of the exchanged sequence was preserved.

**Spacer that separates the two promoter elements follows the rule of six.** An interesting feature of viruses containing a bipartite replication promoter is that the genome length follows the rule of six. Residues important for replication were also found to be located every six nucleotides, possibly due to the encapsidation process by the nucleoprotein N (17). Since we could show that insertion of 4 nucleotides within a region whose sequence was not important for replication abolished replication activity, we were now interested in whether the

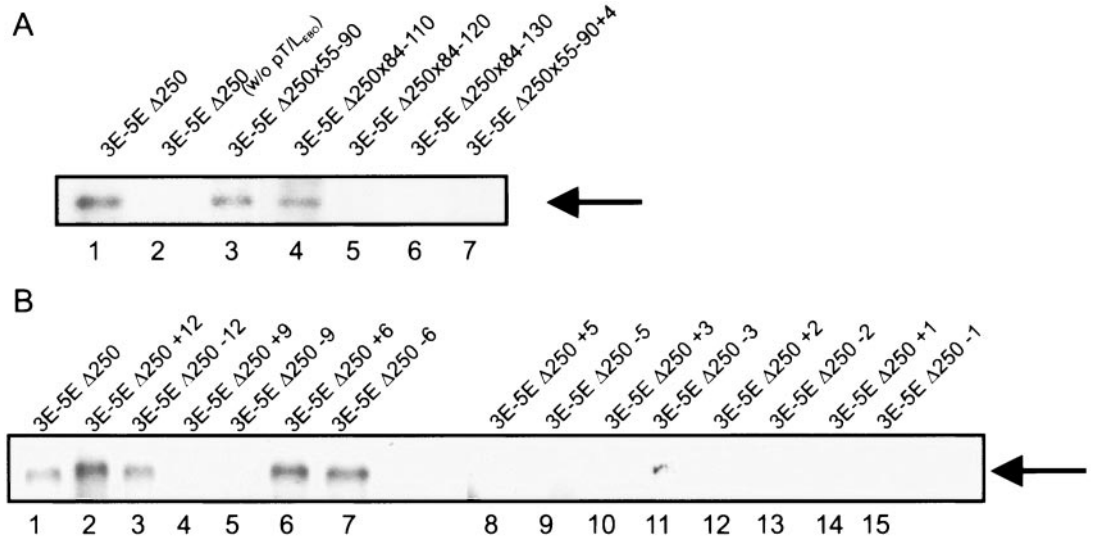


FIG. 5. EBOV replication promoter is bipartite. Huh-T7 cells were transfected with plasmids pT/VP35$_{EBO}$, pT/NP$_{EBO}$, and pT/L$_{EBO}$ and the respective minigenome. At two days posttransfection, cells were lysed and the lysates treated with micrococcal nuclease. Replicated RNA was purified and analyzed by Northern blot assay. (A) Extended regions were substituted with random sequences to elucidate the nature of the EBOV replication promoter. Lane 1 shows the wild-type minigenome as a positive control, and lane 2 is a negative control where plasmid pT/L$_{EBO}$ was omitted from transfection. (B) Analysis of the spacer region between the two promoter elements. Nucleotides were either deleted (odd lanes) or inserted (even lanes) to identify the length of the spacer. Sequences were inserted between nucleotides 71 and 82 (see Table 2 for details).

spacer length, i.e., the relative positions of the two promoter elements, was of any importance.

Two types of spacer mutants were designed: deletions of nucleotides between positions 71 and 82 on the one hand and insertions of up to 12 nucleotides after position 73 on the other hand. Since the mutations were introduced using 3E-5EΔ250 as a template containing a functional transcription start signal, CAT activity could be used to obtain preliminary data for replication. Two days after transfection of Huh-T7 cells with the plasmids coding for the nucleocapsid proteins along with the various minigenomes, cells were harvested and either used for analysis in a CAT assay (data not shown) or lysed for detection of replicated RNA. Interestingly, we found a very clear relationship between the length of the spacer region and replication efficiency: when 6 or a multiple of 6 nucleotides were inserted (Fig. 5B, lanes 2 and 6) or deleted (Fig. 5B, lanes 3 and 7), replication activity was similar to that of the control (Fig. 5B, lane 1), while all other changes in length abolished replication completely. It is noteworthy that only one nucleotide difference from the optimal context led to a complete loss of function.

**Second promoter element consists of at least three UN$_5$ hexamers.** The data shown above clearly indicate that the rule of six is applicable for the EBOV genomic replication promoter, although the genome length is not a multiple of six. Emphasis was therefore put on the search for repetitive motifs within the second promoter element. Indeed, several candidates were revealed, mainly by comparison with other members of the *Mononegavirales*. The motif reported for Sendai virus (42), (CN$_5$)$_3$, where N is any nucleotide, is located in the EBOV genome between nucleotides 108 and 125, however, single substitutions of the C residues did not affect replication efficiency. Another promising motif, (CN$_{11}$)$_3$, located at nucleotides 84 to 119, was also found to be insignificant with regard to replication (data not shown).

Further analysis showed eight adjacent hexamers of the structure UN$_5$ (nucleotides 81 to 128), seven of which were even UAN$_4$ (Fig. 7). Subsequent exchange from 3′ to 5′ (viral RNA sense) of these U residues with A, starting with nucleotide 81 was performed by in vitro mutagenesis. As a result, minigenomes with either five, four, three, or two remaining functional hexamers were created (named 3E-5EΔ250 3U→A, 3E-5EΔ250 4U→A, 3E-5EΔ250 5U→A, and 3E-5EΔ250 6U→A, respectively). Huh-T7 cells were transfected with plasmids coding for the nucleocapsid proteins and the minigenomes, and cells were harvested at 2 days posttransfection for analysis of replicated RNA. Northern blot analysis was used to detect positive-stranded replicated RNA (Fig. 6A).

When three U residues at nucleotides 81, 87, and 93 were exchanged, replication was still very efficient (Fig. 6A, lane 1). Substitution of an additional U residue of the upstream hexamer reduced replication significantly (Fig. 6A, lane 3). When five out of the eight hexamers were mutated (UN$_5$→AN$_5$), replication still occurred but was considerably diminished to almost background (Fig. 6A, lane 5). Two adjacent hexamers were no longer able to support replication of the minigenome (Fig. 6A, lane 7). These findings were supported by the results of CAT assays (Fig. 6B). Since replication activity is a prerequisite for reporter gene expression (see above), these results confirmed that 3E-5EΔ250 5U→A is still active. As a conclusion, at least three adjacent hexamers with the structure UN$_5$ were essential for the second promoter element of the EBOV replication promoter. This indicates that the second promoter element starts at nucleotide 81, where the first U of the hexamers is located. Furthermore, our data revealed that the more

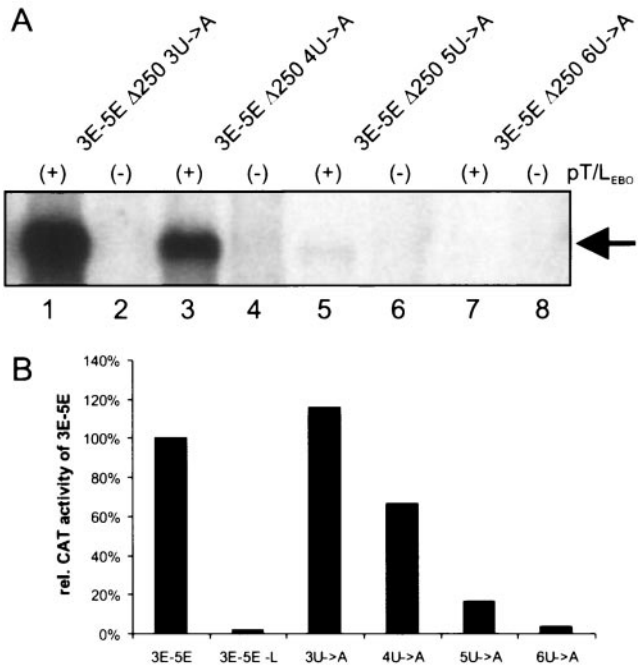


FIG. 6. Substitutions of the putative second promoter element. In total, eight adjacent $UN_5$ hexamers are present within nucleotides 81 to 128 of the EBOV 3′ end. Starting at nucleotide 81, U residues were consecutively exchanged to A (3E-5EΔ250 3U→A contains five hexamers, 3E-5EΔ250 6U→A only two). (A) Northern blot analysis. Huh-T7 cells were transfected with plasmids encoding the nucleocapsid proteins $L_{EBO}$, $VP35_{EBO}$, and $NP_{EBO}$ and the minigenomes as indicated above the lanes. At 2 days posttransfection, cells were harvested and lysed and the lysates were treated with micrococcal nuclease. Replicated RNA was purified and analyzed by Northern blot assay. The arrow indicates the position of replicated RNA. (B) CAT assay. Huh-T7 cells were transfected with plasmids encoding the nucleocapsid proteins $L_{EBO}$, $VP35_{EBO}$, $VP30_{EBO}$, and $NP_{EBO}$ and the minigenomes as indicated. At 24 h posttransfection, cells were probed for CAT activity and acetylated chloramphenicol was quantified. The values refer to 3E-5E as control (100%). As a negative control, the L gene was omitted (3E-5E −L).

hexamers of the given structure were present, the more efficiently replication occurred.

Taken together, our data showed that the first 90 nucleotides were not able to support replication but replication reached almost wild-type levels when 98 nucleotides were present. This led to the assumption that the second promoter element lies roughly between nucleotides 90 and 100. However, substitution of nucleotides 84 to 110 did not affect replication (Fig. 5A, lane 4), indicating that any three adjacent $UN_5$ hexamers can function as (part of) the second promoter element. When the 3′ end was at least 93 nucleotides in length, three hexamers with a U at positions 81, 87, and 93 remained (Fig. 7). Randomizing nucleotides 84 to 110 left three functional hexamers with U residues at positions 111, 117, and 123 to serve as the second promoter element, whereas mutant 3E-5EΔ250 ×84–120 contained only two adjacent hexamers. Therefore, these data can be used to explain the discrepancy of our previous findings that the minimal sequence determined by deletion analysis (Fig. 4A, lane 11) could be exchanged with a random sequence (Fig. 5A, lanes 3 and 4) without any major effect on replication.

## DISCUSSION

In this paper, the EBOV replication promoter was analyzed. We could show that the promoter is bipartite in nature, consisting of a first element located within the first 55 nucleotides of the EBOV genome and a second, downstream element separated by a spacer region spanning at least 25 nucleotides. The second element is located in the 3′ NTR of the first gene and consists of eight $UN_5$ hexamer repeats. However, three consecutive hexamers, which could be located anywhere within the promoter element, were sufficient to support replication as long as the hexameric phase was preserved.

Bipartite promoters have been described for a variety of viruses belonging to the *Paramyxovirinae* subfamily (15, 16, 24, 33, 42, 49). A common feature of these viruses is that their genome length has to be a multiple of six to be replicated efficiently (6, 8, 37, 40). This requirement of a hexameric genome length is reflected by the structure of the replication promoters, consisting of two elements separated by a spacer. Thus, it is postulated that both promoter elements must be positioned along the same face of the helical nucleocapsid, which is achieved by encapsidation of the RNA genome by nucleoprotein (N) subunits, with each N subunit interacting with exactly six nucleotides (48).

The first promoter element of the *Paramyxovirinae* is located

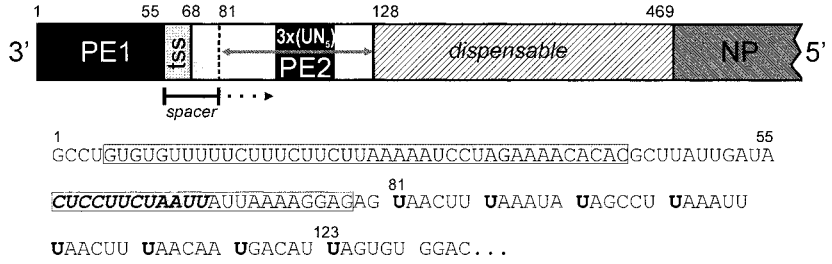


FIG. 7. Proposed structure of the EBOV replication promoter. The two parts of the replication promoter (promoter elements [PE]) are represented by black boxes; the leader (nucleotides 1 to 55) is overlaid by PE1. The second PE consists of eight $UN_5$ hexamers spanning nucleotides 81 to 128 (white box). Three $UN_5$ hexamers are sufficient to support replication and can be dislocated within PE2 in the proper phase. The U residues have to be at position $81 + n \times 6$ (e.g., 81, 87, and 93). PE1 and PE2 are separated by a spacer region ranging at least from nucleotides 56 to 80, including the transcription start signal (tss) of the NP gene. Most of the 3′ nontranslated region of the NP gene is not important for replication or transcription (striped box, nucleotides 129 to 469). The nucleotide sequence of the EBOV genomic 3′ end is depicted under the scheme. The transcription start signal of the NP gene is in italics and bold; nucleotides involved in secondary-structure formation are boxed; and hexameric U residues are in bold.

at the very 3′ end of the genome and spans approximately 30 nucleotides. The second promoter element of the *Paramyxovirinae* is well defined and consists of three adjacent hexamers with the structure $CN_5$ for the respiroviruses Sendai virus and human parainfluenza virus type 3 (16, 42) and for the morbillivirus measles virus (49). A similar $(N_4CG)_3$ motif was identified for the rubulavirus simian virus 5 and for the avulavirus Newcastle disease virus (24, 33). The second promoter element of the *Paramyxovirinae* comprises either hexamers 13 to 15 or hexamer subunits 14 to 16 (47).

Interestingly, the second EBOV promoter element starts at nucleotide 81, which is located within hexamer 14. Despite the requirement for a hexameric phase within the second promoter element of EBOV, its structure seems to be different from that of the *Paramyxovirinae* promoters. First, conserved C or G residues essential for replication could not be identified. Instead, U residues at every sixth position within the promoter element were found to be critical for replication activity. Second, the downstream element of EBOV comprises eight hexamers. However, three hexamers in line located in the proper phase were sufficient for replication activity, although our data suggest that replication occurred more efficiently when more hexamers were present. Finally, and most importantly, the genome length of EBOV is not divisible by six. Neither the full-length genome with 18,959 nucleotides nor naturally occurring defective interfering particles were found to be a multiple of six or another common integer (5, 32). The lack of an integer-length rule is one of the common features of filoviruses and members of the *Pneumovirinae*, such as RSV (38).

Sequence comparison revealed that filoviruses are more closely related to RSV than to any other viruses (30). Moreover, in contrast to the *Paramyxovirinae*, transcription of both RSV and EBOV is dependent on the presence of the viral transcription activator protein M2-1 and VP30, respectively (2, 50). However, the replication promoter of RSV has been shown to consist of a single element located entirely within the leader region (25), indicating that, despite the close relationship between RSV and EBOV, the structure of the replication promoters is different.

Although the rule of six is valid for all members of the *Paramyxovirinae*, strict adherence is required only for respiro- and morbilliviruses, whereas rubula- and avuloviruses are more flexible (48). The EBOV genome length does not obey the rule of six, although hexamer phasing appears to be important for a functional promoter.

The identified minimal spacer region of the EBOV promoter comprises the transcription start signal of the NP gene (12 nucleotides) and the following 13 nucleotides. Exactly this region has been shown to form a stem-loop structure which is involved in regulation of VP30-dependent transcription (50). Although the highly conserved transcription start signal of the first gene has been found to be essential for transcription initiation, this region is not important for replication. Interestingly, the stretch of U residues with a hexamer periodicity is interrupted by those nucleotides which are needed for secondary structure formation at the first gene start site (Fig. 7, nucleotides 56 to 78, second gray box), suggesting an at least partial separation of signals relevant for transcription and replication. For the members of the *Paramyxovirinae* subfamily, it is also known that the transcription start signal does not belong to the replication promoter but to the spacer region. Interestingly, synthesis of the first *Paramyxovirinae* mRNA species always starts at nucleotide 56 (47). This is also the case for EBOV subtypes Zaire and Reston, but not for Marburg virus.

The identified $UN_5$ hexamers within the EBOV genomic replication promoter are found not only with the subtype Zaire but also with EBOV Reston and Marburg virus. Depending on the frame, a stretch of five or six $UN_5$ subunits is located downstream of the EBOV Reston transcription start signal. Similarly, in the Marburg virus 3′ end, the $UN_5$ hexamer is repeated six or seven times and extends into the transcription start signal of the NP gene. Interestingly, the respective motif is also found within the cTrailer of EBOV Zaire and Reston and Marburg virus. Here, the stretch of hexamers comprises four subunits (Marburg virus) and five subunits (EBOV Reston and Zaire). So far, it is not known if the identified $UN_5$ motifs are relevant for EBOV Reston and Marburg virus replication or if they are part of the antigenomic promoters. Further studies will reveal their significance for replication.

It is likely that besides the identified U residues, other nucleotides are also important for replication. Thus, it is noteworthy that each of the U residues is followed by two purine residues, mostly AA (Fig. 7). In Table 2, the sequence and the hexamer phase of the spacer mutants are shown. Interestingly, in mutant 3E-5EΔ250 −5, a stretch of three $UN_5$ hexamers is still in the correct phase. Nevertheless, this mutant was silent, suggesting that additional motifs are relevant for replication. In contrast to the active mutants in which 6 or 12 nucleotides were inserted or deleted, the U residues in mutant 3E-5EΔ250 −5 are not followed by two purines, suggesting that the purine residues might belong to the *cis*-acting signals needed for replication. However, further studies are required to clarify the importance of these nucleotides for replication.

The first EBOV promoter element comprises the leader region from nucleotides 1 to 55 (Fig. 7). We found a stable secondary structure formed internally within the leader by base pairing of nucleotides 5 to 15 with 35 to 44, including a loop of 19 nucleotides (Fig. 2). Formation of the detected RNA structure was independent of the presence of the 5′ end, indicating that a panhandle structure is not formed between the 3′ and the 5′ end. The structure determined by chemical modification assays differed only slightly from the computer prediction shown by Crary et al. (7). Disruption of the stem by exchanging residues $A_{10–13}$ to U abolished replication, and compensatory mutations ($U_{36–39}$→A) could not restore replication (Fig. 3B). This led to the conclusion that secondary-structure formation is not sufficient to support replication, whereas the primary sequence is crucial for promoter function.

These data are in line with those reported by Crary et al. (7), who could show that substitution of single residues within the predicted hairpin loop did not affect replication dramatically. When they destabilized the secondary structure by exchanging three nucleotides, replication was still detectable. However, one of these residues ($U_{16}$) was not involved in the formation of the stem, according to our data (Fig. 2B). But our data agree with theirs that this secondary structure is not the main determinant of replication. For other NNS viruses, it has also been described that RNA secondary structures do not play a discernible role in replication (15). In contrast, panhandle structures occur in segmented negative-stranded RNA viruses such

as influenza A virus and were shown to be involved in interaction with the viral polymerase (10–12). In the case of a positive-stranded RNA virus such as hepatitis C virus, secondary structures are important regulatory elements of viral functions such as translation and replication (22, 44).

It has to be noted that all structures determined experimentally were obtained from naked in vitro-transcribed RNA in the positive-sense orientation due to the methodological limitations of the assay. Primer extension analysis on negative-strand RNA could not be performed because of the requirement for a primer binding site. Moreover, attempts to subject isolated EBOV nucleocapsids to chemical modification assays have not yet been successful. Thus, it remains unclear whether encapsidated RNA is able to form secondary structures. Minigenome RNA has been found to be resistant to nuclease treatment, suggesting a close association of nucleocapsid proteins and RNA. A possible explanation could be that the short time that the polymerase accesses the RNA template is sufficient for secondary-structure formation.

Interestingly, RNA folding is not observed only at the extreme genome ends. Thus, the transcription start signal of all filoviral genes is predicted to form a stable stem-loop structure (31). The function of one of these structures, the stem-loop formed by the transcription start signal of the NP gene and downstream sequences, has been elucidated. This structure is found only 11 nucleotides downstream of the hairpin structure within the leader (Fig. 7) and is known to regulate VP30-dependent transcription, an important process in the viral life cycle (50). However, in contrast to the hairpin loop within the leader region, folding of the second stem-loop could take place on the mRNA level, i.e., on the level of naked RNA.

## ACKNOWLEDGMENTS

We thank Angelika Lander for excellent technical assistance.

This work was supported by the Boehringer Ingelheim Fonds (to M. Weik), by the FAZIT Stiftung and the Fonds der Chemischen Industrie (to S. Enterlein), and by the Deutsche Forschungsgemeinschaft (SFB 535).

S. Enterlein performed this work in partial fulfillment of the requirements for a Ph.D. degree from the Philipps-Universität Marburg, Marburg, Germany.

## REFERENCES

1. **Becker, S., C. Rinne, U. Hofsass, H. D. Klenk, and E. Mühlberger.** 1998. Interactions of Marburg virus nucleocapsid proteins. Virology **249:**406–417.
2. **Bermingham, A., and P. L. Collins.** 1999. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. Proc. Natl. Acad. Sci. USA **96:**11259–11264.
3. **Boehmann, Y., S. Enterlein, A. Randolf, and E. Mühlberger.** 2005. A reconstituted replication and transcription system for Ebola virus Reston and comparison with Ebola virus Zaire. Virology **332:**406–417.
4. **Buchholz, U. J., S. Finke, and K. K. Conzelmann.** 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. **73:**251–259.
5. **Calain, P., M. C. Monroe, and S. T. Nichol.** 1999. Ebola virus defective interfering particles and persistent infection. Virology **262:**114–128.
6. **Calain, P., and L. Roux.** 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J. Virol. **67:**4822–4830.
7. **Crary, S. M., J. S. Towner, J. E. Honig, T. R. Shoemaker, and S. T. Nichol.** 2003. Analysis of the role of predicted RNA secondary structures in Ebola virus replication. Virology **306:**210–218.
8. **Durbin, A. P., J. W. Siew, B. R. Murphy, and P. L. Collins.** 1997. Minimum protein requirements for transcription and RNA replication of a minigenome of human parainfluenza virus type 3 and evaluation of the rule of six. Virology **234:**74–83.
9. **Fearns, R., P. L. Collins, and M. E. Peeples.** 2000. Functional analysis of the genomic and antigenomic promoters of human respiratory syncytial virus. J. Virol. **74:**6006–6014.
10. **Flick, R., and G. Hobom.** 1999. Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation. J. Gen. Virol. **80:**2565–2572.
11. **Flick, R., G. Neumann, E. Hoffmann, E. Neumeier, and G. Hobom.** 1996. Promoter elements in the influenza vRNA terminal structure. RNA **2:**1046–1057.
12. **Fodor, E., D. C. Pritlove, and G. G. Brownlee.** 1994. The influenza virus panhandle is involved in the initiation of transcription. J. Virol. **68:**4092–4096.
13. **Halpin, K., B. Bankamp, B. H. Harcourt, W. J. Bellini, and P. A. Rota.** 2004. Nipah virus conforms to the rule of six in a minigenome replication assay. J. Gen. Virol. **85:**701–707.
14. **Hardy, R. W., and G. W. Wertz.** 1998. The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. J. Virol. **72:**520–526.
15. **Hoffman, M. A., and A. K. Banerjee.** 2000. Analysis of RNA secondary structure in replication of human parainfluenza virus type 3. Virology **272:**151–158.
16. **Hoffman, M. A., and A. K. Banerjee.** 2000. Precise mapping of the replication and transcription promoters of human parainfluenza virus type 3. Virology **269:**201–211.
17. **Iseni, F., F. Baudin, D. Garcin, J. B. Marq, R. W. Ruigrok, and D. Kolakofsky.** 2002. Chemical modification of nucleotide bases and mRNA editing depend on hexamer or nucleoprotein phase in Sendai virus nucleocapsids. RNA **8:**1056–1067.
18. **Jahrling, P. B., T. W. Geisbert, N. K. Jaax, M. A. Hanes, T. G. Ksiazek, and C. J. Peters.** 1996. Experimental infection of cynomolgus macaques with Ebola-Reston filoviruses from the 1989–1990 U.S. epizootic. Arch. Virol. Suppl. **11:**115–134.
19. **Kolakofsky, D., P. Le Mercier, F. Iseni, and D. Garcin.** 2004. Viral DNA polymerase scanning and the gymnastics of Sendai virus RNA synthesis. Virology **318:**463–473.
20. **Kolakofsky, D., T. Pelet, D. Garcin, S. Hausmann, J. Curran, and L. Roux.** 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. J. Virol. **72:**891–899.
21. **Li, T., and A. K. Pattnaik.** 1999. Overlapping signals for transcription and replication at the 3′ terminus of the vesicular stomatitis virus genome. J. Virol. **73:**444–452.
22. **Lytle, J. R., L. Wu, and H. D. Robertson.** 2001. The ribosome binding site of hepatitis C virus mRNA. J. Virol. **75:**7629–7636.
23. **Mahanty, S., and M. Bray.** 2004. Pathogenesis of filoviral haemorrhagic fevers. Lancet Infect. Dis. **4:**487–498.
24. **Marcos, F., L. Ferreira, J. Cros, M. S. Park, T. Nakaya, A. Garcia-Sastre, and E. Villar.** 2005. Mapping of the RNA promoter of Newcastle disease virus. Virology **331:**396–406.
25. **McGivern, D. R., P. L. Collins, and R. Fearns.** 2005. Identification of internal sequences in the 3′ leader region of human respiratory syncytial virus that enhance transcription and confer replication processivity. J. Virol. **79:**2449–2460.
26. **Miranda, M. E., M. E. White, M. M. Dayrit, C. G. Hayes, T. G. Ksiazek, and J. P. Burans.** 1991. Seroepidemiological study of filovirus related to Ebola in the Philippines. Lancet **337:**425–426.
27. **Modrof, J., S. Becker, and E. Mühlberger.** 2003. Ebola virus transcription activator VP30 is a zinc-binding protein. J. Virol. **77:**3334–3338.
28. **Modrof, J., E. Mühlberger, H. D. Klenk, and S. Becker.** 2002. Phosphorylation of VP30 impairs Ebola virus transcription. J. Biol. Chem. **277:**33099–33104.
29. **Mühlberger, E., B. Lötfering, H. D. Klenk, and S. Becker.** 1998. Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. J. Virol. **72:**8756–8764.
30. **Mühlberger, E., A. Sanchez, A. Randolf, C. Will, M. P. Kiley, H. D. Klenk, and H. Feldmann.** 1992. The nucleotide sequence of the L gene of Marburg virus, a filovirus: homologies with paramyxoviruses and rhabdoviruses. Virology **187:**534–547.
31. **Mühlberger, E., S. Trommer, C. Funke, V. Volchkov, H. D. Klenk, and S. Becker.** 1996. Termini of all mRNA species of Marburg virus: sequence and secondary structure. Virology **223:**376–380.
32. **Mühlberger, E., M. Weik, V. E. Volchkov, H. D. Klenk, and S. Becker.** 1999. Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. J. Virol. **73:**2333–2342.
33. **Murphy, S. K., and G. D. Parks.** 1999. RNA replication for the paramyxovirus simian virus 5 requires an internal repeated (CGNNNN) sequence motif. J. Virol. **73:**805–809.
34. **Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka.** 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J. Virol. **76:**406–410.
35. **Pattnaik, A. K., L. A. Ball, A. LeGrone, and G. W. Wertz.** 1995. The termini of VSV DI particle RNAs are sufficient to signal RNA encapsidation, replication, and budding to generate infectious particles. Virology **206:**760–764.

36. **Peters, C. J., and A. S. Khan.** 1999. Filovirus diseases. Curr. Top. Microbiol. Immunol. **235:**85–95.
37. **Rager, M., S. Vongpunsawad, W. P. Duprex, and R. Cattaneo.** 2002. Polyploid measles virus with hexameric genome length. EMBO J. **21:**2364–2372.
38. **Samal, S. K., and P. L. Collins.** 1996. RNA replication by a respiratory syncytial virus RNA analog does not obey the rule of six and retains a nonviral trinucleotide extension at the leader end. J. Virol. **70:**5075–5082.
39. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. USA **93:**3602–3607.
40. **Skiadopoulos, M. H., L. Vogel, J. M. Riggs, S. R. Surman, P. L. Collins, and B. R. Murphy.** 2003. The genome length of human parainfluenza virus type 2 follows the rule of six, and recombinant viruses recovered from nonpolyhexameric-length antigenomic cDNAs contain a biased distribution of correcting mutations. J. Virol. **77:**270–279.
41. **Sutter, G., M. Ohlmann, and V. Erfle.** 1995. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. FEBS Lett. **371:**9–12.
42. **Tapparel, C., D. Maurice, and L. Roux.** 1998. The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: a motif $(GNNNNN)04_3$ is essential for replication. J. Virol. **72:**3117–3128.
43. **Thomas, S. M., R. A. Lamb, and R. G. Paterson.** 1988. Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. Cell **54:**891–902.
44. **Thurner, C., C. Witwer, I. L. Hofacker, and P. F. Stadler.** 2004. Conserved RNA secondary structures in Flaviviridae genomes. J Gen. Virol. **85:**1113–1124.
45. **Volchkov, V. E., S. Becker, V. A. Volchkova, V. A. Ternovoj, A. N. Kotov, S. V. Netesov, and H. D. Klenk.** 1995. GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology **214:**421–430.
46. **Volchkov, V. E., V. A. Volchkova, E. Mühlberger, L. V. Kolesnikova, M. Weik, O. Dolnik, and H. D. Klenk.** 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science **291:**1965–1969.
47. **Vulliemoz, D., S. Cordey, G. Mottet-Osman, and L. Roux.** 2005. Nature of a paramyxovirus replication promoter influences a nearby transcription signal. J Gen. Virol. **86:**171–180.
48. **Vulliemoz, D., and L. Roux.** 2001. "Rule of six": how does the Sendai virus RNA polymerase keep count? J. Virol. **75:**4506–4518.
49. **Walpita, P.** 2004. An internal element of the measles virus antigenome promoter modulates replication efficiency. Virus Res. **100:**199–211.
50. **Weik, M., J. Modrof, H. D. Klenk, S. Becker, and E. Mühlberger.** 2002. Ebola virus VP30-mediated transcription is regulated by RNA secondary structure formation. J. Virol. **76:**8532–8539.
51. **Yu, H., C. W. Grassmann, and S. E. Behrens.** 1999. Sequence and structural elements at the 3′ terminus of bovine viral diarrhea virus genomic RNA: functional role during RNA replication. J. Virol. **73:**3638–3648.